U.S. Patent Application No. 304 481

D E C L A R A T I O N

We, the undersigned Gabrijela Kobrehel and Slobodan Djokić, hereby declare that we have carried out the following comparative tests, whose results are attached to this Declaration:

Teble 3: Testing of in vitro potency of N-methyl-11-aza-10-deoxo-10-dihydroerythromycin A in comparison with erythromycin A and its 11-aza derivative against 182 gram-positive bacterial organisms

Table 4: Testing of in vitro potency of N-methyl-11-aza-10-deoxo-10-dihydroerythromycin A in comparison with erythromycin A and its 11-aza derivative against 179 gram-negative bacterial organisms

Table 5: Testing of in vitro sensitivity of 30 anaerobic bacterial organisms against N-methyl-11-aza-10-deoxo-10-dihydroerythromycin A in comparison with erythromycin A and its 11-aza derivative

Table 6: Testing of acute toxicity in mice by the method of Litchfield-Wilcoxon of N-methyl-11-aza-10-deoxo-10-dihydroerythromycin A in comparison with erythromycin A and its 11-aza derivative

Table 7: Testing of acid stability at pH 1.2 of N-methyl-11-aza-10-deoxo-10-dihydroerythromycin A, 2'-propionyl-N-methyl-11-aza-10-deoxo-10-dihydroerythromycin A,

N-methyl-11-aza-10-deoxo-10-dihydroerythromycin A cyclic 13,14-carbonate, and 2'-acetyl-N-methyl-11-aza-10-deoxo-10-dihydroerythromycin A cyclic 13,14-carbonate in comparison with the parent erythromycin A, by exposure of the compounds to hydrochloric acid (pH 1.2) and determination of the residual activity by two-fold dilution technique vs. Staphilococcus aureus ATCC 6538-P and expressing the results in minimum inhibitory concentrations (MIC) in mcg/ml

Figure 1: Blood level studies in rabbits after oral doses of 100 and 250 mg of N-methyl-11-aza-10-deoxo-10-dihydroerythromycin A (1) in comparison with the oral administration of 250 mg of erythromycin A

The undersigned further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or any patent issuing thereon.

Zagreb, January 31, 1984 Gabrijela Kobrehel
Slobodan Đonić

Table 3. Antibacterial in vitro activity on gram-negative positive clinical isolates

TEST ORGANISM	COMPOUND	MIC (mcg/ml) 0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	128.0	R	No of tested strains
Enterococcus	E	26	11	9		6	3		1		7	
	A	20	6	11	1	5	4		4		12	63
	1	15	13	10	6				4		15	
Staph. aureus	E	9	7	12							13	
	A	10	7	12	1			1			10	41
	1	11	10	4	6	3					7	
Staph. albus	E	22	6	12		2						
	A	24	9	4	2	3						42
	1	30	12									
Streptococcus pneumoniae	E	9	7									
	A	12	4									16
	1	11	5									
Streptococcus haemolyticus	E	17	3									
	A	20										
	1	13	7									
No. of sensitive strains	E	83	34	34		8	3		1		20	182
	A	86	26	26	4	8	4	1	4		22	182
	1	80	47	14	12	3			4		12	182

E - Erythromycin A

A - 11-Aza-10-deoxo-10-dihydroerythromycin A

1 - N-Methyl-11-aza-10-deoxo-10-dihydroerythromycin A

R - resistant

Table 4. Antibacterial *in vitro* activity on gram-negative clinical isolates

TEST ORGANISM	COMPOUND	MIC (mcg/ml) 0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	128.0	R	No.
	E					2	28	40	8		22	100
E. coli	A	1		2	39	50	2	1	2		3	
	1		4	42	45	5	2	1	1		0	
	7		2	37	53	3		1	2		2	
	E						1	2	2		4	9
Klebsiella	A				3	1	3	1			1	
pneumon.	1				4	4	1					
	7					2	4	1	2			
	E						1		2		7	10
Klebsiella	A					3	5				2	
aerogenes	1					9		1				
	7					5	1	3			1	
	E					13					3	16
Proteus	A					2		3	1		10	
mirabilis	1				1	2	1	10			2	
	7				2		1		6		7	
	E								1		9	10
Pseudomo-	A					1		2	3		4	
nas aerug.	1					1		1	5		3	
	7						1	4	1		4	
	E								1		17	18
Enterobac-	A					1	5	7	4		1	
ter aerog.	1				3	6	6	2			1	
	7					3	9	1	5			
	E						1					1
Enterobac-	A					1						
ter liquef.	1				1							
	7					1						
	E						1				2	3
Mima	A							2	1			
polymorpha	1					1		1	1			
	7					1	1	1				

Continued on the next page:

Table 4 (continued)

TEST ORGANISM	COMPOUND	MIC (mcg/ml) 0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	128.0	R	No.
Herella	E					1	3	1	1			6
	A		1				1	3			1	
	1		1	1			3	1				
	7			1	2	2			1			
Haemophylus influ.	E	1		2	3							6
	A	1	3	2								
	1	3	3									
	7	1	4	1								
No. sensitive strains	E	1	0	2	3	16	35	45	15		64	179
	A	2	4	4	42	59	16	19	11		22	179
	1	3	8	43	54	28	13	17	7		6	179
	7	1	6	39	57	17	17	11	17		14	179

E - Erythromycin A

A - 11-Aza-10-deoxo-10-dihydroerythromycin A

1 - N-Methyl-11-aza-10-deoxo-10-dihydroerythromycin A

7 - N-Methyl-11-aza-10-deoxo-10-dihydroerythromycin A 13,14-cyclic carbonate

Method: Two-fold serial dilution technique in MH agar.

No. - number of tested strains

R - resistant

Table 5. Sensitivity of anaerobic bacteria

TEST ORGANISMS	COMPOUND	MIC (mcg/ml)										No. of tested str.
		0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	128.0	R	
BACTEROIDES FRAGILIS	E			1	3	2						6
	A		1	4	1							
	1	3		3								
BACTEROIDES NECROPHORUS	E			2	2	2						6
	A	3	1	2								
	1	2	3	1								
BACTEROIDES MELANINOGENICUS	E			2		1						3
	A	1	1	1								
	1	1	2									
VEILONELLA SP	E		2	1	1							4
	A	3		1								
	1	4										
LEUCONOSTOC	E		3									3
	A	2	1									
	1	2	1									
PEPTOSTREPTOCOCCUS	E	5	3									8
	A	7	1									
	1	8										
No. of sensitive strains	E	5	8	6	6	5						30
	A	16	5	8	1							30
	1	20	6	4								30

E - Erythromycin A

A - 11-Aza-10-deoxo-10-dihydroerythromycin A

1 - N-Methyl-11-aza-10-deoxo-10-dihydroerythromycin A

Method: Two-fold serial dilution technique in MH agar.

R - resistant

Table 6. Acute toxicity

Compound[+]	Route of administration	LD_{50}	LD_{16}	LD_{84}
E	I.V.	360 (241.9)[++]	280	360
	I.P.	520 (349.4)	326	840
	P.O.	-	-	-
A	I.V.	280 (141.7)	220	350
	I.P.	-	-	-
	P.O.	> 10000 (5060)		
1	I.V.	825 (421.6)	610	1020
	I.P.	1200 (513.2)	1010	1400
	P.O.	> 10000 (5100)		

E - Erythromycin A

A - 11-Aza-10-deoxo-10-dihydroerythromycin A

1 - N-Methyl-11-aza-10-deoxo-10-dihydroerythromycin A

[+] The substances were tested as the lactobionate salts.
[++] The values calculated in the relation to the active compound.

Table 7. Acid stability at pH 1.2 (MIC in mcg/ml)

Exposure time	MIC				
in hours	E	1	5	7	8 +
control-without					
acid	0.1	0.1	0.1	0.5	0.5
0	5	0.1	0.1	0.5	0.5
1/2	10	0.1	0.1	0.5	0.5
1	10	0.5	0.5	0.5	0.5
3	25	1.0	0.5	1.0	0.5
6	25	2.5	0.5	0.1	0.5

E = Erythromycin A

1 = N-Methyl-11-aza-10-deoxo-10-dihydroerythromycin A

5 = 2'-Propionyl-N-methyl-11-aza-10-dexo-10-dihydroerythromycin A

7 = N-Methyl-11-aza-10-deoxo-10-dihydroerythromycin A cyclic 13,14-carbonate

8 = 2'-Acetyl-N-methyl-11-aza-10-dexo-10-dihydroerythromycin A cyclic 13,14-carbonate

Strain: Staphylococcus aureus ATCC 6538-P

+ Arabic figures correspond to the notation of the Examples

Figure 1. Blood level studies in rabbits after oral doses of 100 and 250 mg